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Changes of Glycoprotein Patterns in Sera of Humans under Stress

Karmela Barišić[1], *Gordan Lauc*[1], *Jerka Dumić*[1], *Mladen Pavlović*[2] and *Mirna Flögel*[1]

[1] Department of Biochemistry and Molecular Biology, Faculty of Pharmacy and Biochemistry, University of Zagreb, Zagreb, Croatia
[2] Institute for Medical Research and Occupational Health, Zagreb, Croatia

Summary: Stress exhibits adverse effects on many vital processes in which glycoproteins play a significant role (e. g. cell-cell/matrix interactions, immune response, neoplastic growth, implantation, prenatal development), yet only scarce attention has been directed towards studying stress induced changes in glycoprotein patterns. Using SDS-electrophoresis, blotting and digoxigenin-labelled lectins (*Sambucus nigra* agglutinin, *Galanthus nivalis* agglutinin, *Datura stramonium* agglutinin, *Maackia amurensis* agglutinin and peanut (*Arachis hypogaea*) agglutinin), sera were analysed from 30 individuals chosen randomly from a severely stressed population of 309 male volunteers with no specific medical symptoms. Significant changes were found in glycoprotein pattern and content, compared with healthy controls of matching age and sex. Occasionally minor non-specific deviations from the reference values for several analytes (haemoglobin, glucose, bilirubin and alanine aminotransferase) were detected in the tested group, but glycoprotein GP45 (M_r = 45 000), detected by *Datura stramonium* agglutinin and *Sambucus nigra* agglutinin, appeared in 96.7% of samples of the stressed population. The same population also revealed an approximately 500-fold increase of GP37 in comparison with the control sera. These results suggest that stress, as a non-specific syndrome, induces specific biochemical changes, which could be of diagnostic relevance as risk makers before any more serious symptoms of stress-related consequences have developed.

Introduction

Glycoproteins are molecules which contain more structural information than any other molecules in the human body. According to the current knowledge of the underlying biosynthetic machinery, the structural carbohydrate complexity arises from the multiplicity of enzymes involved in their synthesis (1) suggesting functional significance of the information stored in the carbohydrate parts of glycoproteins (2, 3).

Glycoproteins are known to play a key role in many vital processes from fertilization, blastocyst implantation and prenatal development, to immune response and cancer (4). Evidence for their involvement in the stress response is growing (5, 6) and recently it was even demonstrated that some oligosaccharide structures can have beneficial effects in murine traumatic shock (7).

Stress and depressive symptoms have been associated with the development and course of many human diseases from simple virus infections to cancer (8, 9). Many of these diseases can be associated with a stress-induced decrease in the immune reponse, and a notable number of studies have addressed this problem (10, 11). Significant effects have been found in the stress-induced suppression of natural killer cytotoxicity (12), and production of specific antibodies (13).

Exposure to stress during pregnancy have severe adverse consequences for child development. When rats were exposed to the foot-shock or swimming stress during early pregnancies, a significant decrease of body weight gain and increased offspring mortality were detected (14, 15). When stress was experienced during the pre-implantation stage, it prevented implantation and caused complete abolition of pregnancy (16, 17). Epidemiological studies in humans have also indicated that psychological stress causes a significant increase of habitual abortions, lower birth-weight and a higher rate of pregnancy abnormalities (18–20).

In this study we analysed changes in the sera of individuals who underwent intensive stress while imprisoned in war camps of former Yugoslavia. The life conditions in these camps are reported to have been harsh (21, 22) and a number of medical (23), psychological (24) and immunological (25) problems were present among detainees (tab. 1). Standard biochemical quantities were also analysed (tab. 2). Deviations from the standard values were found in some detainees, but none of the tests proved to be a common and specific marker (26, 27).

As mentioned above, glycoproteins have an important role in many stress-related processes. However, only few studies have been undertaken to evaluate stress-re-

lated changes of specific glycoproteins and lectins (28–34).

Lectins, natural specific receptors for glycoproteins are useful tools for specific detection and characterization of glycoproteins in vitro (35, 36). We used a set of five specific lectins listed in table 3 to identify individual glycoproteins in normal (control) sera and in sera from detainees.

Materials and Methods

Materials

5-Bromo-4-chloro-3-indolylphosphate, *p*-nitro blue tetrazolium and bovine serum albumin were purchased from Sigma (St. Louis, MO), Immobilon PVDF membrane from Millipore (Bedford, MA), digoxigenin-labelled *Galanthus nivalis*, peanut (*Arachis hypogaea*), *Sambucus nigra*, *Maackia amurensis and Datura stramonium* agglutinins (lectins), and alkaline phosphatase-conjugated anti-digoxigenin $F(ab)_2$ fragments from Boehringer Mannheim (Mannheim).

Study population

Sera from individuals, imprisoned for at least 90 days in detention camps in the area of former Yugoslavia (eastern Slavonia), were examined after their release in March 1992. For this study 30 detainees (males, age 19–45) were selected randomly from the group of 309 volunteers (informed consent) with no apparent infectious or other disease (or significant physical injuries) detected by medical examination. Sera were donated willingly by detainees within three days after release from camps and stored at −80 °C until analysed. The control group (n = 9) of sera was taken from apparently healthy volunteers (of matching age and geographic origin).

Tab. 1 Health problems in war prisoners liberated from concentration camps (23, 26)

Problem	Frequency (%)
Rash	5
Albuminuria	7
Leukocyturia	6
Headaches	63
Dizziness	70
Disturbed sleep	57
Nightmares	32

Tab. 2 Deviations form normal biochemical values in prisoners of war (26)

Result	Percentage of
Decreased haemoglobin (< 139 g/l)	24%
Increased bilirubin (> 20 µmol/l)	13%
Increased creatinine (> 110 µmol/l)	8.2%
Increased alanine aminotransferase (> 35 IU/l)	19%
Decreased glucose (< 3 mmol/l)	0.7%
Increased glucose (> 6 mmol/l)	6.9%

Tab. 3 Specificity of lectins

Lectin	Source	Specificity
GNA	*Galanthus nivalis*	Man-α(1,3), Man-α(1,6), or Man-α(1,3)-Man
SNA	*Sambucus nigra*	Sia-α(2,6)-Gal
MAA	*Maackia amurensis*	Sia-α(2,3)-Gal
PNA	Peanut (*Arachis hypogaea*)	Gal-β(1,3)-GalNAc
DSA	*Datura stramonium*	Gal-β(1,4)-GlcNAc

Electrophoresis and blotting

Serum proteins were separated electrophoretically in 12% SDS-polyacrylamide slab gels as described by *Laemmli* (37). After electrophoresis, proteins were transferred onto Immobilon PVDF membranes in a semi-dry apparatus (Pharmacia, Sweden) according to *Towbin* et al. (38). After blotting, membranes were blocked overnight with 30 g/l bovine serum albumin, and developed with digoxigenin-labelled lectins in the following dilutions: 1 : 1000 for *Sambucus nigra*, *Galanthus nivalis* and *Datura stramonium* agglutinins, 1 : 200 for *Maackia amurensis* agglutinin and 1 : 100 for peanut agglutinin (35). The formed glycoprotein-lectin complexes were detected with anti-digoxigenin $F(ab)_2$ fragments conjugated with alkaline phosphatase and visualized with 0.02 g/l 5-bromo-4-chloro-3-indolylphosphate and 0.04 g/l nitro blue tetrazolium in 50 mmol/l Tris/HCl, pH = 8.5, 100 mmol/l NaCl, 5 mmol/l $MgCl_2$.

Amounts of individual glycoproteins were calculated by scanning and integrating spots on the membrane. Integration was performed with GelScan XL software (Pharmacia) by fitting *Gaussian* curves into scanning results on the basis of horizontal background. Intensities of individual glycoprotein bands were expressed in relative units as defined in the GelScan software (area units).

Protein concentrations were determined using the biuret method (39).

Results

Galactose-specific peanut agglutinin, mannose specific *Galanthus nivalis* agglutinin, and sialic acid-specific *Maackia amurensis* agglutinin did not show any significant differences in the resulting binding patterns between sera from detainees and control individuals (fig. 1).

In contrast, *Sambucus nigra* and *Datura stramonium*-reactive glycoproteins in the sera of detainees showed

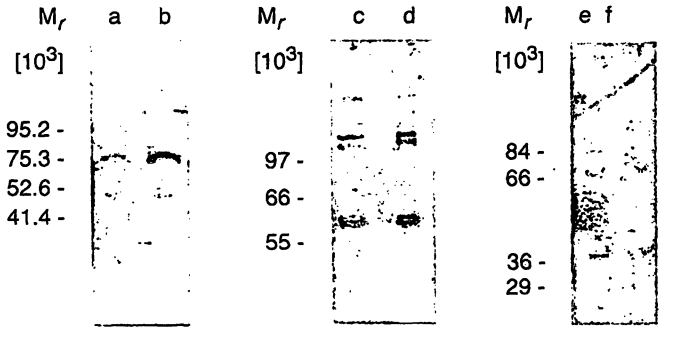

Fig. 1 Patterns of glycoproteins in control sera (a, c, e) and in sera from individuals under stress (b, d, f). Serum proteins (20 µg of total protein per well) were separated on 12% SDS PAGE, transferred to Immobilon PVDF membrane and detected with *Galanthus nivalis* agglutinin (a, b), *Maackia amurensis* agglutinin (c, d), and peanut agglutinin (e, f). There were no differences in the glycoproteins reactive with these three lectins which could be correlated with exposure to stress. The double band which sometimes appeared with the *Galanthus nivalis* agglutinin appeared with similar frequencies in both populations.

Tab. 4 Relative amounts[a] of stress induced glycoproteins. Presented values are averages from 30 stress-samples (detainees) and 9 control individuals. Standard deviations are shown in parentheses.

Glycoprotein M_r	Detection with *Datura stramonium* lectin		Detection with *Sambucus nigra* lectin	
	Stress	Control	Stress	Control
37 000	<0.01 (−)	<0.01 (−)	3.533 (0.986)	0.087 (0.059)
45 000	0.645 (0.231)	<0.01 (−)	2.107 (0.574)	<0.01 (−)

[a] Amounts of glycoproteins are expressed in the relative units (area) as defined in the Pharmacia GelScan® software.

significant alterations in comparison with control sera (tab. 4, figs. 2 and 3). A random example of a normal lectin-binding pattern for Gal-β(1,4)-GlcNAc specific *Datura stramonium* agglutinin is shown on figure 2b. Only one major glycoprotein band, of apparent mass of 50 000, is visible. Figure 2a shows a typical example of a *Datura stramonium* agglutinin-binding pattern in sera from detainees. In addition to the 50 000 band, there is an another clearly visible glycoprotein with an apparent molecular mass of 45 000. The incidence of appearance of this glycoprotein (GP45) in samples originating from detainees exceeded 96% (found in 29 out of 30 detainees). GP45 was completely absent from all examined control sera, thus suggesting that it is induced by stress.

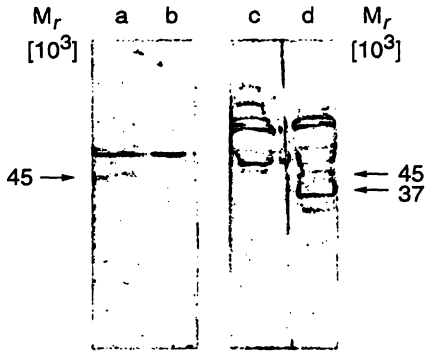

Fig. 2 An example of stress-induced changes of lectin binding patterns in sera from a detainee (a, d) compared to a control individual (b, c). Serum proteins (20 μg of total protein per well) were separated on 12% SDS PAGE and transferred to Immobilon PVDF membrane. Glycoproteins were detected using *Datura stramonium* (a, b) and *Sambucus nigra* agglutinin (c, d). Two major alterations were detected: (i) appearance of a novel M_r = 45 000 band (GP45) which was detected with both *Datura stramonium* and *Sambucus nigra* agglutinin; and (ii) 500-fold increase in concentration of M_r = 37 000 *Sambucus nigra* agglutinin-reactive glycoprotein (GP37).

Analysis of serum glycoproteins by Sia-α(2,6)-Gal specific *Sambucus nigra* lectin (fig. 2) also revealed significant alterations in detainees (d) compared with the control group (c). Although more glycoproteins are recognized by *Sambucus nigra* agglutinin than by *Datura stramonium* agglutinin, two distinct changes are clearly visible. The most pronounced one is the appearance of an additional glycoprotein (M_r = 45 000), which probably corresponds to the above mentioned *Datura stramonium* agglutinin-reactive glycoprotein of the same size. Comparison with the normal lectin-binding pattern also showed a significant (more than 500-fold) increase in the amount of the glycoprotein of approximate molecular mass 37 000 (GP37) in sera of detainees (tab. 4, figs. 2 and 3).

Discussion

In the presented study we analysed glycoprotein patterns in sera from normal individuals and from individuals

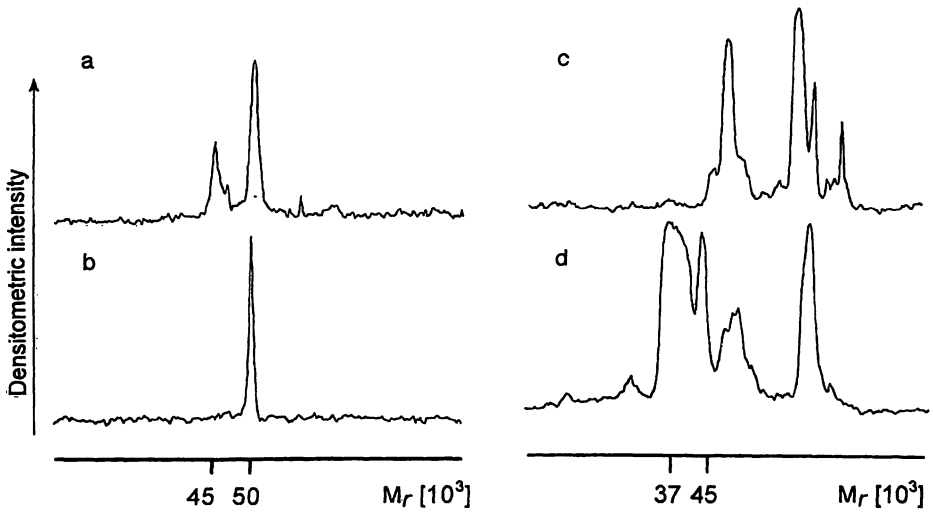

Fig. 3 Comparisons of *Datura stramonium* (a, b) and *Sambucus nigra* agglutinin (c, d) lectin binding patterns in sera from stressed (a, d) and control subject (b, c). The figure represents densitometric scans of the blots from figure 2. For detailed explanation see Materials and Methods.

detained in war prisoner camps for at least 90 days, being exposed to complex inescapable stress including food deprivation, intense fear, and possible maltreatment. Our aim was to find a specific biochemical response of the human organism to this kind of stress. Previously reported results of basic biochemical tests showed deviations from normal values in some detainees, but none of the analysed properties was shown to be a specific marker present in all detainees (26, 27).

We found two major changes of serum glycoproteins in nearly all detainees (96.7%):

(i) appearance of a novel $M_r = 45\,000$ glycoprotein (figs. 2 and 3); and

(ii) a 500-fold increase in the amount of GP37.

Both of these glycoproteins were virtually absent from all control sera and present in all but one analysed detainee serum. The nearly ubiquitous presence of these glycoproteins in detainees, irrespectively of their physical status, individual variability, differences in treatment and medical history, and absence of the same glycoproteins in control individuals, strongly indicate that these glycoproteins are a consequence of exposure to intense stress.

On the basis of lectin-binding patterns, and the assumption that 45 000 glycoproteins recognized by *Sambucus nigra* agglutinin and *Datura stramonium* agglutinin are the same protein, it is possible to partly identify the carbohydrate parts of these glycoproteins. GP45 was recognized by galactose-specific *Datura stramonium* agglutinin, and by sialic acid-specific *Sambucus nigra* agglutinin, while it was not recognized by mannose-specific *Galanthus nivalis* agglutinin, galactose-specific peanut agglutinin (galactose linked in a different way from the recognized by *Datura stramonium* agglutinin), and sialic acid-specific *Maackia amurensis* agglutinin (sialic acid linked in a different way from that recognized by *Sambucus nigra* agglutinin). It seems that GP45 contains branched oligosaccharides with at least two different termini. One terminus is characterized by galactose, possibly bound by a β(1,4) glycosidic linkage to N-acetyl-glucosamine, but certainly not through a β(1,3) linkage to N-acetyl-galactosamine. Another terminus possesses sialic acid, probably bound to galactose through a α(2,6) glycosidic linkage. Lack of recognition of this glycoprotein by the mannose-specific *Galanthus nivalis* agglutinin implies that it does not contain terminal mannose residues and is not a high-mannose glycoprotein.

On the basis of the positive reaction of GP37 with *Sambucus nigra* agglutinin and its negative reaction with *Galanthus nivalis* agglutinin, GP37 is also not a high-mannose glycoprotein and contains at least one terminus with sialic acid, probably bound to galactose through a α(2,6) glycosidic linkage. Further studies aimed at the purification of GP37 and GP45 and the determination of their structure and possible functions are in progress.

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Corresponding author: Mirna Flögel, Department of Biochemistry and Molecular Biology, Faculty of Pharmacy and Biochemistry, University of Zagreb, Domagojeva 2, HR-1000 Zagreb, Croatia